

The University of Chicago

A MICROPOPULATION STUDY OF *EUGLENA*
GRACILIS KLEBS IN STERILE, AUTO-
TROPHIC MEDIA AND IN BAC-
TERIAL SUSPENSIONS

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

1937

By

HELEN ELIZABETH SWEET

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A MICROPOPULATION STUDY OF EUGLENA GRACILIS KLEBS IN STERILE, AUTOTROPHIC MEDIA AND IN BACTERIAL SUSPENSIONS¹

(Five figures)

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I. INTRODUCTION

ROBERTSON'S hypothesis of allelocatalysis has prompted a number of experimentalists to check the relations of numbers of organisms, volumes, and rates of division in order to test his proposition that "there is acceleration of multiplication by the contiguity of a second organism in a restricted volume of nutrient medium" (Robertson, 1924, p. 1242). This may be called the "Robertson effect." Robertson's hypothesis to account for this type of acceleration has been summarized clearly by Allee (1931, p. 166):

During nuclear division each nucleus retains the charge of autocatalyst with which it was provided, and adds to it during the course of nuclear synthesis. At each division the autocatalyst is shared between the nuclear substance and the surrounding medium in a proportion determined by its relative solubility and by its affinity for chemical substances within the nucleus. The mutually accelerative or allelocatalytic effect of contiguous cells is due to each cell's losing less of the autocatalyst to the medium because of the presence of the other.

Robertson's observations suggest some of the interrelationships existing between members of the same species of relatively asocial animals and plants which are known to affect the structural and functional characteristics of such individuals and even the integration of their communities. Tests of the reality of his theory have been made primarily with protozoans.

The work of several careful experimentalists has given evidence to support Robertson's phenomenon, if not his hypothesis, although even more work with numerous species of protozoans under various conditions tends to uphold the findings of Woodruff (1911), which are exactly opposite. These studies, both for and against the Robertson effect, are thoroughly reviewed by Allee (1931, 1934) and by Johnson (1937). Johnson (1936), whose work with *Oxytricha* and *Paramecium* has included the relations between reproductive rates of different numbers of individuals seeded in different volumes and varying densities of bacterial suspensions, has shown, among other things, that in the presence of sparse suspensions of the bacterium *Pseudomonas fluorescens*, cultures seeded with isolated individuals reproduced faster; with dense suspensions those seeded with two individuals showed greater reproductive rates. Gause (1934) terms this the "Johnson effect" and produces independent evidence in its support.

The relation and interrelation between numbers, food, and volume for each species of

¹ I am gratefully indebted to Dr. W. C. Allee, who suggested this problem to me and whose kindly advice and untiring interest have made the completion of this paper possible after an extended data-finding period and whose help in evaluating and clarifying the results has been invaluable.

animal and plant present in a microcosm is a complex problem, particularly when bacteria and other microorganisms are involved. Johnson (1937) also summarizes much of this work.

These experiments indicate that the number of individuals with which a culture is inoculated is not an absolute factor by means of which the ensuing division-rates of ciliates may be predicted but that with different species, in certain volumes and conditions, seeding a culture with greater numbers insures a faster initial division-rate, and vice versa; the greater numbers generally show relatively greater division-rates in larger volumes and/or when the bacterial content is high.

Such results resemble those which Robertson discovered and assigned to the allelo-catalytic effect of a nuclear X-substance given off into the medium during cell division, but these later results can be accounted for in terms of other quantitative relations which exist between the medium and the special organisms studied. There is a recent revival of the original theory in the reports of Karl Reich (1938) with an amoeba, *Mayorella palestinensis*, and of Mast and Pace (1938), with *Chilomonas paramecium*, in which the observed results apparently cannot be attributed wholly to nutrient-organism relations.

Much work has been done on the laboratory ecology of *Euglena gracilis*, which I shall not cite here, in view of the fact that there are extensive reviews and bibliographies to be found in such papers as those of Jahn (1934, 1935a), Dusi (1930, 1936), Mainx (1928), Zumstein (1900), and Dangeard (1901).

Since *E. gracilis* is an autotroph, there is opportunity, by limiting the bacteria, to check the effects of numbers of the same species in specific media more closely than with the ciliates or with other heterotrophic organisms.

Experimental work varying the numbers of *E. gracilis* in the inoculum has been attempted only by Jahn (1929, 1930). In a few isolation cultures he found no consistency in division-rates in small volumes. He attempted 2- and 6-drop cultures (size of drop not stated), which were inoculated with single individuals after two washings, details of which are not given. They were followed over a 10-day period. As a result of this experience he concluded that this method was impractical, owing to changes in salt concentrations from evaporation of small volumes over such a period of time and because of surface-interior relations which are not identical with those of cultures with larger volumes.

In mass cultures Jahn (1929) did not obtain absolutely sterile conditions but considered an initial bacterial count of 3,000 per cubic centimeter and a final count of 3,000,000 per cubic centimeter an inappreciable contamination. This number, however, may be responsible, at least in part, for the shape of his growth curves.

With one *Euglena* in from 383,300 to 353,300,000 times its volume of culture media, Jahn found that (1) division-rates decrease as the number of seeded organisms increases; (2) the division-rates decrease as time increases; and (3) the number of organisms increases in time and then decreases. As Jahn says (1930), an increase in numbers, taken alone, is not necessarily a test of an autocatalytic effect in a long-time study (cf. Morgan [1926] and Snell [1929]). Deaths aside, in counts taken at the end of the first 24 hours, however, the division-rate is indicated fairly directly by the number of individuals present.

Jahn (1929) found from mass cultures that the smaller the initial number of organisms per volume, the greater the numbers produced at the end of a 6- or 10-day period. In 48 hours the difference was not great but was slightly in favor of those cul-

tures inoculated with the greatest number of individuals. Without more information one can point out only that this acceleration in the second 24-hour period is what might be expected from either the Robertson or the Johnson effects, so called.

The present paper attempts to explore further the problem of interorganismic effects on the division-rate of *E. gracilis*. Experimental conditions include various number and volume relations, depression-slide cultures, mass flask cultures (not discussed here), relatively sterile cultures employing dilution and irradiation methods, and cultures in controlled bacterial suspensions. The experiments have been made to test the role of numbers of the same species and of controlled populations of associated bacteria with respect to specific volume relations as factors determining division-rates.

Preliminary experiments, using various techniques and types of infusion, showed no consistent results with reference to number in the inocula. These cultures contained such a large number of variables that an attempt was made to set up a series of systems in which the environmental conditions were more nearly under control.

The effects of different numbers in the inocula are mainly investigated, although certain cross-comparisons between growth in different cultures will be given. In this paper the following aspects of the problem are discussed:

1. A comparison of division-rates of cultures inoculated with one, two, four, and eight individuals in several small volumes, using dilution methods for obtaining relatively bacteria-free euglenae.
2. A similar comparison when the euglenae are freed from bacteria by exposure to ultra-violet irradiation.
3. Similar comparisons in suspensions of controlled concentrations of mixed or pure cultures of bacteria when the euglenae are freed from bacteria by both of the foregoing methods.
4. The effects of numbers present on survival after long exposure to ultra-violet irradiation.
5. A comparison of the results with these small-volume cultures with those of Jahn's mass cultures.

II. CULTURE METHODS

A clone of *E. gracilis* was isolated at the beginning of the summer of 1934 from a culture obtained from the General Biological Supply House, Chicago. The first clone served for all the cultures of 1934 and 1935. A second clone, established in the spring of 1936 and used for work of that summer, was found to be no different statistically in division-rates or any other measured characteristic from Clone I. This clone was isolated from a culture furnished by the Albert Galigher Laboratories of Berkeley, California.

The standard experimental medium throughout was the salt solution of Osterhout (1906), as modified by Barker and Taylor (1931), made with block-tin and then glass-distilled water and using analyzed grades of the following salts in a liter of water: 7.8 gm. $\text{MgCl}_2 \cdot \text{H}_2\text{O}$; 3.8 gm. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2.2 gm. KCl ; 1.0 gm. CaCl_2 ; and 1.0 gm. NaCl . This was diluted 500 times with double-distilled water before autoclaving and using. One cubic centimeter of $\text{M}/20 \text{ NaH}_2\text{PO}_4$ was used to buffer each 30 cc. of the distilled solution, and pH adjustments were made with $\text{M}/20 \text{ NaOH}$.

Ferric chloride was added to each liter of the salt solution before using it, so that there was a concentration of 0.00002 per cent ferric chloride per liter. Unlike usual practice in flagellate culturing, no acetate, citrate salts, or organic material was used, since a medium

containing as little organic matter as possible was desired. Glucose, Leibig's extract, Difco tryptophane, peptone, or muscle extracts are added by most recent experimentalists, such as Jahn (1929-35), Alexander (1931), Hall (1933), and Elliott (1938). While several students of chlorophyll-bearing organisms, such as Zumstein (1900) and Ternetz (1912), reported unfavorable growth in an autotrophic medium, Pringsheim (1912) and Mainx (1928), working with both heterotrophic and autotrophic media, conclude that growth of flagellates was as good in an autotrophic as in a heterotrophic medium. They do not, however, present evidence concerning the degree of bacteriological sterility of their media. Jahn used the inorganic salt media of Mainx (1928) and Gunther (1928), to which he added hydrolyzed casein for most of his work. This salt solution also had either ammonium or potassium nitrate salts as nitrogen sources. Dusi (1933) succeeded in culturing several species of *Euglena*, using inorganic sources of nitrogen, and later (1936, 1937) carried through four transfers, at least, in a totally inorganic medium. Dusi also finds that growth is much facilitated when organic materials are present. Mast, Pace, and Mast (1936) report success in growing *Chilomonas*, a supposed saprozoite, on an inorganic medium, while Hall and Loefer (1936) failed to grow this species when the dilution of tryptone was beyond 1:100,000.

The solution used in the present work lacks nitrogen in quantities determinable by sulphanilic nitrite or Nessler's ammonium tests. However, the reagents used have minute quantities of nitrogen present as impurities; and, likewise, the distilled water shows traces of it. The question of nitrogen-fixation on the part of *Euglena* is entirely problematical. There was no evidence from tests that the nitrogen content increased in cultures in sterile salt solution in a 24-hour period. It is possible that sufficient nitrogen is stored within each organism to supply several generations, or the disintegration of one or more of the original individuals in a culture may furnish enough of this substance. The amount of organic stuff in a 1:500,000 dilution of the stock medium must have been an extremely meager source of nitrogen or other essential organic matter. This lack of available nitrogen may account for slower division-rates in cultures started with one or two individuals in relatively large volumes when compared with those given with larger numbers in the inoculum.

The present results concur with those of Dusi (1937), Hutner (1936), Jahn (1935a), Hall and Schoenborn (1939), and others, in that growth was not as abundant or as assured in an inorganic medium as in infusions of several types. Cessation, and finally death, was a common result from cultures in salt solutions in large volumes. This inability of cultures to continue growth regularly in sterile inorganic salt media or to attain maximal numbers in mass cultures in such media, even with bacteria present, undoubtedly is reflected in the early losses in isolation cultures.

Stock cultures grew abundantly in wheat infusions which were made by boiling 10 grains of dried wheat in 100 cc. of distilled water for 10 minutes and allowing it to stand 24 hours before being inoculated with several drops from an established culture. Ten thousand *Euglena* per pipette drop (0.05 cc.)² was a typical count in such infusions.

The original stock culture was developed from an isolated individual which produced twelve euglenae in 24 hours. The division-rates of subclones were carefully studied to detect possible variation in cultures with small volumes. In wheat infusions, where bacterial contamination and evaporation are great, no variation in division-rates, which

² Unless specifically stated to the contrary, the drops used in this study contained approximately 0.05 cc.

were statistically significant, appeared in 26 hours. In 36, 42, and 66 hours significant variations did occur. Readings at the end of 24 hours only are used in this paper; hence no significant variation is to be expected as a result of differences inherent in the euglenae themselves.

In preparation of any series for an experiment the following dilution methods were employed to reduce the number of bacteria. This method was used for all experiments unless otherwise designated as sterilized by exposure to ultra-violet irradiation, as described at the beginning of Section IV.

One drop of a stock culture, approximately 2 weeks old, was mixed with 5 cc. of autoclaved salt solution in a sterile watch glass³ and was allowed to stand 1 hour. This is called "dilution Bath A": it diluted the stock 101 times. Bath B was made by transferring 1 drop of Bath A to 10 drops of sterile salt solution, diluting the stock 1,111 times. Euglenae were kept in this bath 10 minutes. Bath C involved a transfer of 5 micropipette drops (0.0025 cc.), each microdrop containing one *Euglena*, to 5 large drops (0.05 cc.); and this mixture was allowed to stand 20 minutes. This last bath brought the total dilution of the stock medium to 23,331, and the concentration of the stock became approximately 0.00004.

As euglenae were transferred from Bath C to sterile salt-solution cultures of from 1 to 40 drops, the cases of greatest concentration of the stock present in any of the experiments would occur with eight euglenae present in 1 drop. This further dilution would contain approximately 0.000002 concentration of the stock materials of bacteria.

These changes in concentration were gradual enough to avoid shock, since there was no readily apparent change in motility or morphology.

Bacterial counts (Table 1), employing plating methods as used in standard water analyses, were taken at various intervals in the dilution series. Counts made at the end of the first 8 minutes in Bath C showed fewer bacteria in the salt solution than at the end of 10 or 12 minutes, after which time there was no increase. Tests made at the end of 15 minutes in Bath C, according to standard bacteriological methods, rarely showed more than seven viable bacteria present per cubic centimeter. Later in the present paper, when cultures are said to be "sterile" or "bacteria-free," this degree of sterility is meant.

Stock cultures tended to become alkaline, pH 8.1-8.5, whereas the sterile salt solution was buffered to a pH of 7.6. Subcultures in small drops kept the same pH at least 24 hours. The change from stock-culture media of greater alkalinity apparently offered no great physiological hazard, although this may have been one of the reasons that reproductive rates were greatest in wheat solutions.

Because of the use of carbon dioxide by autotrophs, carbonic acid does not accumulate. If CO₂ utilization is a measure of division-rate or of metabolism, the more alkaline media might indicate more complete metabolism; and, likewise, greater acidity might be associated with less complete use of materials in the medium and with comparatively slower division-rates.

Quantitative studies on the optimal pH for the growth of *E. gracilis* in various types of organic media and with various temperature and light relationships have been reported by Kostir (1921), Jahn (1931, 1932, 1935a), Hall (1933), Wang (1928), and others.

Measurement of CO₂ consumption, O₂ production, and oxidation-reduction potentials

³ All glassware was washed with soap and water and rinsed with flowing hot tap water or was allowed to stand in glass-cleaning solution overnight and rinsed thoroughly with running hot tap water and dried with clean linen towels, wrapped in heavy tissue paper and sterilized in a dry oven at 190° C.

for *Euglena* species have not been reported. Jahn (1933) found, with *Chilomonas*, that the reduction of sulphur was very important in establishing high division-rates. Certainly, CO₂ sources are of great importance to a chlorophyll-energy system. Because

TABLE 1
NUMBERS OF COLONIES OF BACTERIA IN 1 CC. OF VARIOUS SALT-SOLUTION
BATHS AND CULTURES OF *Euglena gracilis* EMPLOYING THE
AGAR-PLATE COUNTING METHOD
(Average of ten cases, 1935 and 1936)

Section	Culture	Time Elapsed	Mean	Standard Deviation
A*	Stock	2 wk.	4,330,000.0	500,400.0
	Bath A	60 min.	34,200.0	8,600.0
	Bath B	10 min.	4,600.0	1,200.0
	Bath B	20 min.	8,300.0	2,300.0
	Bath C	8 min.	2.0	1.0
	Bath C	10 min.	3.4	1.0
	Bath C	12 min.	3.3	1.0
	Bath C	15 min.	3.0	0.1
B†	a) Washed sterile cultures in sterile salt solution (8 euglenae present)	10 min.	1.00	0.05
		24 hr.	0.75	0.01
	b) Ultra-violet radiated 2-minute salt-solution culture (8 euglenae present)	10 min.	1.20	0.04
		24 hr.	0.06	0.00
	c) Ultra-violet radiated 3-minute salt-solution culture	10 min.	0.00	0.00
		24 hr.	0.01	0.02
C‡	a) X concentration No euglenae present	10 min.	3,109,700,100	75,000
		24 hr.	3,107,900,100	49,100
	8 euglenae present	10 min.	3,100,600,060	54,200
		24 hr.	3,111,637,940	71,230
	b) 0.001 X concentration No euglenae present	10 min.	2,876,000	4,920
		24 hr.	2,500,900	5,700
	8 euglenae present	10 min.	2,914,912	7,200
		24 hr.	3,241,617	7,140

* Bacterial counts from dilution baths in preparation for washed-sterile euglenae cultures.

† Bacterial counts from 1 drop of washed-sterile, irradiated 2-minute, and irradiated 3-minute cultures with eight euglenae present at the beginning and end of an experiment.

‡ Bacterial counts from *Pseudomonas* suspensions of X and 0.001 X concentrations with and without euglenae present.

division-rate is notably higher in organic media, it is easily surmised that a major function of certain bacteria or of organic radicals may be that of serving as links in the carbon or nitrogen cycles of *Euglena* metabolism.

Temperature has been constant to within 0.5 C. in any one series of experiments.

Temperatures of 23°, 25°, and 27° C. have yielded approximately similar results in 1935, 1934, and 1936, respectively; at least, no significant differences could be found in the average division-rates shown by comparable cultures in these three series.

Constancy of light, humidity, and temperature were maintained by use of a specially constructed chamber, which was operated in a room with controlled temperature and humidity. This chamber, designed by Dr. W. C. Allee, is a cylinder 3 feet in diameter and 3 feet in height with a revolving press-board floor. It had as its source of light six 100-watt "daylight" mazda bulbs, each with a reflector and suspended from an overhanging disk 3 feet above the floor of the chamber. The light passed through a heat screen of over 2 inches of filtered-water plate glass and caused little distortion of the light. During an experiment the chamber was lighted constantly, day and night. The thermograph records of the temperature within the chamber showed the variation to be no greater than 0.5° C. The humidity of the constant-temperature room was under positive control and remained around 67 ± 2 per cent; within the light chamber there was less variation from this mean. It was also possible to place the crystallization dishes holding the depression slides of an experiment in this chamber without any movement of the cultures within a given experimental period.

III. A COMPARISON OF NUMBERS PRODUCED IN CULTURES INOCULATED WITH ONE TWO, FOUR, AND EIGHT INDIVIDUALS IN VARIOUS SMALL VOLUMES

Cultures of *Euglena* (washed free from bacteria, as described in the previous section) showed certain similarities in division-rates dependent on the number of organisms which were placed in a given volume. As always in these experiments, all euglenae were from the same clone. In these particular tests they were cultured in from 1 to 40 drops of fresh sterile medium. Series were arranged so that there were eight individuals isolated singly in eight different depression slides; four slides were inoculated with two organisms each; two slides similarly were seeded with four and two others with eight individuals each. One of the last served as a control on which bacteriological tests were made.

This set of sixteen slides constituted one experimental series. Forty individuals were required to inoculate such a series. Each organism was equally and separately washed and was finally transferred to its respective slide within a half-hour of other members of the series. Each slide was covered with a thin cover slip, which was held in place by a film of vaseline or sterile water. Evaporation was reduced to a minimum by placing the covered slides in a moist culture chamber composed of a large crystallization dish in the bottom of which was about an inch of water; the slides were placed on a white platform above the water. Such a chamber, holding eight depression slides, was transferred to the constant light, temperature, and humidity chamber already described. The two crystallization dishes which housed a single experimental series were kept close together, so that all slides received approximately equal illumination.

The subcultures were left undisturbed until they were removed to be counted at the end of 24 hours. The control slide was tested bacteriologically by the plating method. Contamination could not be determined until another day had elapsed; hence, no experiment was concluded until at least 52 hours after it began. Twenty-four-hour counts of *Euglena* proved most satisfactory; in 48 hours there was evidence of slight evaporation, even though the slides had not been inspected in the meantime.

Depression slides were used for volumes of from 1 to 5 drops; watch glasses were used

for those of 10, 20, and 40 drops. The latter were covered with heavy glass slides made to fit and sealed with vaseline. Methods of sterilizing all glassware were described in Section II of this paper.

The data accumulated in three years of experimentation have been summarized in Figure 1 since there were no significant differences in the results for any one year which could make invalid a treatment of the data as a whole. The graphs plot the average total

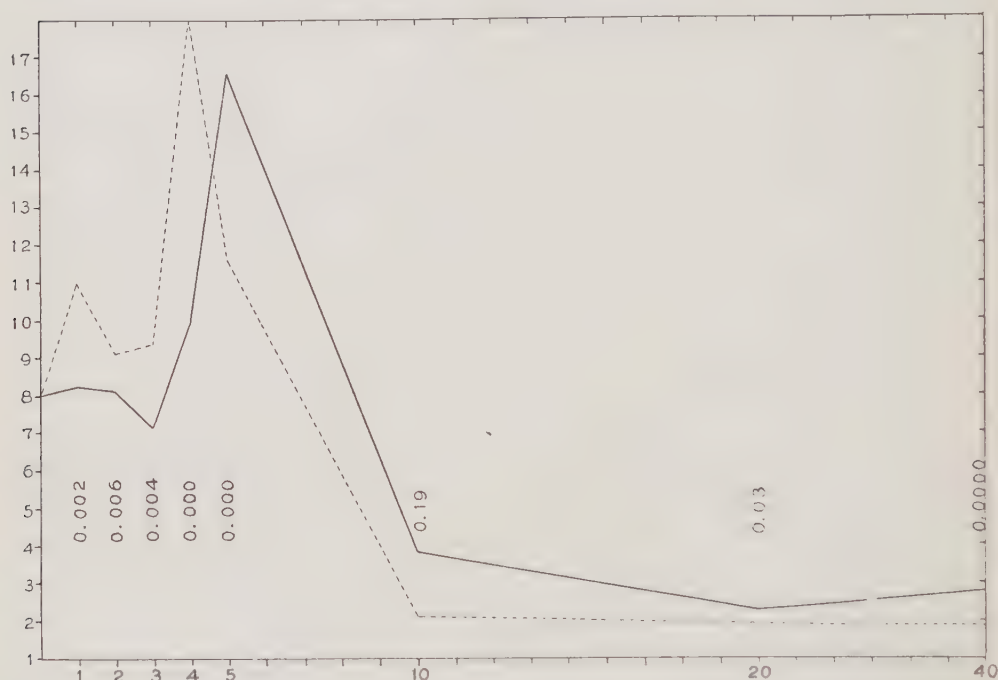


FIG. 1.—Average total numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with one and two (dotted line) contrasted with four and eight (solid line) individuals with statistical probability of the differences. Ordinates, average total numbers present after 24 hours; abscissas, volumes in drops. Numerals give the statistical probability (P) of the observed differences at the several points. This was determined by Student's method for paired experiments. $P = 0.03$ means that there are three chances in one hundred of finding as great differences by random sampling. $P = 0.05$ is usually considered to be the extreme upper limit of statistical significance. The smaller the value of P , the greater the significance.

number of individuals produced in 24 hours in cultures started with one and two euglenae (broken line) and those started with four and eight (solid line) in the various volumes. In addition, the statistical probability of the differences are given for each volume.

Table 2 shows the number of series which have been summarized in Figure 1. The fully expanded table, showing comparisons for the different volumes for the experiments conducted in 1934, 1935, and 1936, summarized by years, is given in detail elsewhere (Sweet, 1937). The summary in Table 2, together with the statistical probabilities shown on the figure itself, gives some indication of the reality of the recorded differences. The

fact that essentially similar results were obtained in three different periods of experimentation adds to their reliability.

In the smaller volumes the cultures started with fewer euglenae reproduce faster than those seeded with four or eight individuals. The mean difference in the average total numbers in the cultures inoculated with one and two euglenae is greater than in those having four and eight in the inocula in 1, 2, 3, and 4 drops; the actual difference is 2.92, with $P = 0.00001$. In 4 drops taken alone, the same cultures also reproduce more rapidly; the difference is 9.5 with $P = 0.0000$.

In 5 drops conditions are reversed; the difference is 6.26 with $P = 0.0000$. In the largest volumes tested, 20 and 40 drops, the average total numbers maintained by the two groups show statistically significant advantage for the larger number in the inocula, with a difference of 1.00 and $P = 0.0000$.

There is very little difference, none of statistical significance, in the average division-

TABLE 2

A SUMMARY SHOWING THE AMOUNT OF EXPERIMENTATION
USED AS A BASIS FOR FIGURE 1

CULTURE VOLUME (IN DROPS)	NUMBER OF SERIES		
	1934	1935	1936
1.....	16	16	9
2.....	16	15	
3.....	13	13	13
4.....	10	3	13
5.....	8	12	
10.....		8	
20.....		11	7
40.....		15	9

rates of each group in the three smallest volumes of 1, 2, and 3 drops, in spite of the fact that the volumes are doubled and tripled. There is, for example, no significant advantage or difference in the numbers produced in cultures started with a single individual in 1 drop, as compared with 2 or with 3 drops of culture medium.

As seen in Figure 1, decided differences in division-rate appear at the transition from 3- to 4-drop cultures; the average rates are doubled, or practically so, for the cultures inoculated with one and two organisms; and there is a 50 per cent acceleration for those started with four and eight individuals. Cultures with one and two euglenae in the inocula attain the highest rates of reproduction to appear anywhere in the series tested. The acceleration in cultures with least numbers in the inoculum, as compared with those with greater, a phenomenon which is typical of the smaller volumes, is greatly enhanced in 4 drops. This volume of 4 drops represents the optimal volume for seedings of one and two euglenae.

In 5 drops the cultures inoculated with the greater numbers show significantly higher reproductive rates, and these reach their maximum in 5 drops. Cultures seeded with one and two individuals decline in division-rates from cultures of 4 drops to those of the 5 drops almost as rapidly as they increased from the smallest volumes to those of 4 drops.

In 4 drops the cultures seeded with eight euglenae had least numbers at the end of 24 hours; in 5 drops these cultures increased the most and attained a maximal number for all volumes tested of those seeded with eight organisms. The optimal volume of cultures inoculated with four and eight individuals is, under the conditions of these experiments, 5 drops; whereas, cultures seeded with one and two individuals reached their maximal numbers in the 4 drops.

In the twenty series tested in 5 drops the cultures inoculated with four and eight individuals gave an average total number of 16.88 at the end of the first 24 hours; similar cultures started with one and two euglenae produced an average of 10.62. The chance of this occurring in random sampling is less than once in several thousand times, $P = 0.0000$.

When identical volume relations obtain per individual in the inoculum and the numbers produced are compared, the cultures started in 5 drops show significantly higher rates than do those of either supraoptimal or suboptimal volumes. One comparison was possible with the suboptimal volumes, and six with the supraoptimal. There were seven cases in which cultures of 4 drops could be contrasted with suboptimal volumes, in which identical volume-per-individual relations existed; and of these, five gave significantly more euglenae in the larger volume. One of the two cases in which the cultures in 4 drops could be compared with supraoptimal volumes showed significantly more organisms produced in 4 drops.

In brief, from this type of comparison it is clear that it is not only the relationship of volume per individual which acts to account for differences in division-rates, but there is, under the conditions of these experiments, a volume which tends to be optimal because it is conducive to greater division-rates almost regardless of the number of inoculated euglenae or initial volume-per-capita relationships.

In 10, 20, and 40 drops (Fig. 1), which constitute the three largest volumes tested, the division-rates fall for all cultures, irrespective of size of inoculum; maintenance of original numbers is impossible; and the cultures seeded with four and eight individuals show significantly greater ability to survive.

In 10 drops considered alone, the cultures with eight initial individuals show significantly greater numbers than do any other cultures; the probability of these differences¹ is in the cases of 1 8 $P = 0.004$, 2 8 $P = 0.0000$, and 4 8 $P = 0.004$. The cultures with four and eight individuals in the inocula, when grouped as in Figure 1, however, do not show significant advantage over those with one and two euglenae seeding a culture in this supraoptimal volume.

In 20 drops there is a slightly significant difference in the division-survival rates of the cultures; four and eight organisms in the inocula make the better showing ($P = 0.03$). In 40 drops cultures with four and eight in the inocula, in 24 parallel cases, show a very reduced but a significantly greater number present after 24 hours than do cultures started with but one or two euglenae.

With the suboptimal volumes one can postulate that one or more of the following factors are acting: (1) too great concentration of excretory products; (2) too great concentration of a growth-promoting substance or substances; (3) too great evaporation and hence a too rapid or too great change in salt concentration or too concentrated an

¹ "1 8" refers to a contrast between the division rate of the cultures inoculated with one organism and those inoculated with eight. The statistical significance of the difference is given as P .

end-product; (4) too great an intake or accumulation of oxygen and far too rapid a loss of carbon dioxide; and (5) too few bacteria or bacterial end-products.

With the supraoptimal volumes the converse of these same conditions holds as possible responsible agents to account for the harmful effects of the greater volumes: (1) too much toxic material per organism; (2) too great dilution of growth-promoting substance or substances; (3) too slow loss of carbon dioxide; (4) too slow intake of oxygen; (5) too slow evaporation, i.e., too slow a change in osmotic pressure; and (6) too many bacteria or bacterial end-products.

In view of the instances in which equal volume-per-organism relations have shown that volume per individual is not, by itself, controlling the division-rates (as explained on p. 182), it is likely that those factors connected with surface-volume differences are those most active in altering the numbers produced or surviving. Points (3) and (4) of both foregoing lists and (5) of the second list are at once subject to surface-volume differences. It is easy to see that in the suboptimal volumes evaporation may play an important role and that, by change in the osmotic pressure of the media, may be deterring division. This is probably the major cause of depression in suboptimal volumes.

On the supraoptimal side of the curve the causal relations are less clear. At present we can only suggest that the relatively increased mass and relatively decreased surface may be associated with the depression, or that the medium is toxic and the toxicity shows up most readily when there is a large volume per individual. This well-established mass effect is discussed at some length by Allee (1931, 1938). As might be expected from similar studies, the toxic effect was less pronounced in cultures seeded with the greater number of euglenae, notably in 10, 20, and 40 drops. Although the average population for such cultures always decreased, differential survival was exhibited in favor of increased numbers in the inocula.

The greatest numbers produced in the small-volume, suboptimal cultures, when contrasted to the numbers maintained in the supraoptimal cultures, is evidence that the conditions obtaining in these small volumes approach the optimal more nearly than do those in the larger volumes for the size of inocula used.

The phenomenon which appears most dramatic in this survey of division-rates of washed-sterile euglenae is the rapid reversal of the size of the inocula which yields maximal numbers. Four and 5 drops represent the optimal volumes for division-rates of euglenae in this type of culture. With the addition of 1 drop of culture media the optimal number of euglenae seeding cultures was changed from the smaller to the greater number in the inocula. It is not clear why this reversal should occur so rapidly and in these particular volumes. However, such a reversal is in line with the experience of certain workers with similar relationships for ciliates, notably Peterson (1929) and Johnson (1933, 1936), except that with their work the transition was less sudden.

Evidence of the Robertson effect or of some modification of it is present in the cultures in 5 drops of these salt-solution series which are relatively free from bacteria. Reproduction and/or survival rates "are accelerated by the presence of contiguous organisms in a limited volume." In the supraoptimal volumes, of 10, 20, and 40 drops, there is certainly a loss of individuals and probably a diminished division-rate in the survivors; even so, more individuals are present in cultures inoculated with greater numbers.

There is no evidence that this effect, even in 5 drops, is to be accounted for by "allelotaxis" as explained by Robertson. The contribution of nitrogen to the media, made possible by the death of one or more individuals in the larger volumes inoculated

with greater numbers, might account for the observed effect. Also, because of the fact that volume per organism is not in all cases a controlling factor, there is evidence that surface-volume factors may be active. More analyses are necessary before the explanation of the observed differences can be established.

IV. EFFECTS OF NUMBERS PRESENT ON DIVISION-RATES OF *Euglena gracilis* FREED FROM BACTERIA BY ULTRA-VIOLET IRRADIATION

Euglenae washed, as before, through baths A and B were irradiated⁵ 2 and 3 minutes in these experiments and were then transferred by micropipette to sterile salt-solution cultures (as explained fully in Sec. II). Bacterial counts taken 10 minutes after transfer showed less than two colonies per 0.05 cc. by plate-counting methods (Table 1, B), and after 24 hours in culture even fewer bacteria were present. The method was, therefore, not any less effective in obtaining practically sterile cultures than the dilution method and involved one less transfer.

The results presented on the effects of irradiation on division-rates are those occurring because of the reactions of 2- and 3-minute irradiated organisms in sterile experimental environments when volumes and numbers of seeded euglenae are the only known variables. Every individual used in these experiments was irradiated in the same volume and with approximately one hundred other euglenae present during the exposure to ultra-violet.

The effects of irradiation for 2 and 3 minutes on populations of various sizes in 1 and 4 drops are shown in Figure 2. The numbers present in these series after 24 hours represent an ability to survive as well as to reproduce, and clearly reveal in these capacities differences which are dependent on the size of the initial population.

It is apparent at the outset that such comparisons as are shown in Figure 2 give evidence that growth of euglenae is not as great following irradiation as after treatment in dilution baths. Hence, we are dealing with an effect which is more harmful than that of washing.

The direction of increase in numbers produced per size of inocula is not the same in the irradiated as in the washed-sterile series, except with 2-minute irradiated euglenae in 4 drops where conditions are least toxic of any of the irradiated series. In 4 drops, washed-sterile euglenae divided significantly more rapidly than did 2-minute irradiated euglenae; in the cultures started with two individuals the value of P for the difference in rate was 0.0000; with four individuals in the inocula, 0.0000; with eight, 0.001; and with one and two considered together, 0.000.

The most harmful effects occurring in these series of irradiated euglenae are in those grown in 1 drop after 3 minutes of irradiation. Three-minute irradiation is decidedly more deleterious than that for 2 minutes, when the euglenae are cultured in either of the two volumes studied. In fact, in cultures in 1 drop and with irradiation for 3 minutes (Fig. 2, *c*) there is a population decrease in 24 hours in the cultures seeded with one, two, and four euglenae; and survival only is measurable. An increase in numbers present occurs only in the cultures with the largest number (eight) in the inocula. In this series an increase in density of initial population is, in every instance, of advantage: statistical

⁵ A Cooper-Hewitt quartz mercury-vapor lamp was used; it ran at 110 volts D.C. and about 4 amperes, and at 66 cm. distance from the center of the arc to the culture being irradiated. It was over 10 years old and had been much used.

significance is attached to each difference in the numbers surviving or produced in cultures with successively greater numbers in the inocula. Table 3 gives the results of a comparison of the differences.

A significant advantage is also accorded larger-sized inocula in the group of experiments of 1 drop with euglenae irradiated for 2 minutes. The value for P for differences

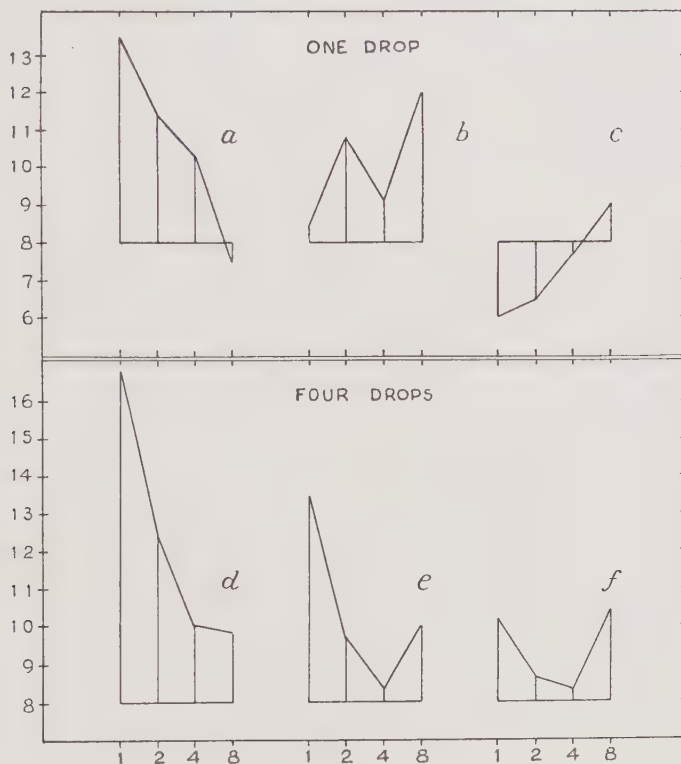


FIG. 2.—Differences in numbers of *Euglena gracilis* present after 24 hours in cultures inoculated with washed-sterile or 2- and 3-minute irradiated euglenae in 1 and 4 drops with varying numbers in the inocula. Ordinates, average total numbers present after 24 hours; abscissas, numbers in the inocula. One drop: (a) washed-sterile euglenae; (b) 2-minute irradiated; (c) 3-minute irradiated. Four drops: (d) washed-sterile; (e) 2-minute irradiated; (f) 3-minute irradiated.

between cultures started with two, as compared with one, individual is 0.04; for 4/1 is 0.0000; and for (4 and 8)/(1 and 2) P is 0.013.

In the larger volume, 4 drops, all cultures are capable of increasing their numbers; and the larger inocula are not significantly more successful than they were in the smaller volumes. Rather, the least numbers in the inocula have a relatively accelerated reproductive rate when only 2-minute irradiation is used, and the differences in the division-rates between smaller- and larger-sized inocula are significant (Table 4).

With euglenae irradiated for 3 minutes and cultured in 4 drops no difference is significant for any size of inocula, and the smaller inocula are not significantly retarded in

this volume. The larger volume is less deterring, therefore, to division of euglenae irradiated 2 or 3 minutes; and the final conditions approach those found in similar cultures started with washed-sterile individuals.

The fact that more harmful effects are seen in the 1-drop series, with either 2- or 3-minute irradiation, than in 4 drops, is in accord with the findings with the washed-sterile series in which optimal culture conditions seemed to prevail in 4 and 5 drops. However, in comparison of population densities in these two volumes, there are effects

TABLE 3
COMPARISON OF AVERAGE NUMBERS PRESENT WITH VARIOUS
SIZED INOCULA OF EUGLENAE IRRADIATED FOR
3 MINUTES AND CULTURED IN 1 DROP

Comparison of Inocula Size	Average Total Numbers Present	Statistical Probability
2/1.....	6.5/6.0	0.000
4/1.....	7.6/6.0	0.02
8/1.....	9.0/6.0	0.0000
4/2.....	7.6/6.5	0.0051
8/2.....	9.0/6.5	0.0000
8/4.....	9.0/7.6	0.000
(1 and 2)/(4 and 8).....	12.5/16.6	0.0000

TABLE 4
COMPARISON OF AVERAGE NUMBERS PRODUCED WITH VARIOUS
SIZED INOCULA OF EUGLENAE IRRADIATED FOR
2 MINUTES AND CULTURED IN 4 DROPS

Comparison of Inocula Size	Average Total Numbers Produced	Statistical Probability
1/2.....	13.5/9.7	0.0098
1/4.....	13.5/8.3	0.0004
1/8.....	13.5/10.0	0.0002
2/4.....	9.7/8.3	0.0000
8/2.....	9.7/10.0	0.0098
8/4.....	9.7/8.3	
(1 and 2)/(4 and 8).....	23.2/18.3	0.0032

on division-rate or on survival which indicate that some factors may be operating which are also dependent on the volume-per-individual phenomena.

In brief, under the conditions of these particular experiments, toxicity or deleterious factors in general, as manifested by lessened division-rates, are most significant in small volumes and when least numbers compose the inocula. As volume increases and or numbers in the inocula increase, such factors show diminished effects.

V. THE EFFECTS OF NUMBERS INOCULATED ON RATE OF DIVISION IN CULTURES WITH SEVERAL SPECIES OF BACTERIA OF KNOWN CONCENTRATION

The following series of experiments were arranged in parallel fashion in order to ascertain the effects of symbionts and so compare the reproductive rates of *E. gracilis* in

the presence of mixed bacteria in known concentration with those given in bacteria-free inorganic media.

The media for these experiments were prepared in the following manner. Stock cultures were made in 100 cc. of the usual salt solution in small Erlenmeyer flasks. These were seeded with one or more pipettefuls of euglenae from the standard wheat-infusion stock and were well contaminated with bacteria of the wheat cultures. One cubic centimeter of supernatant fluid obtained by centrifuging 10 cc. of these salt-solution stock cultures was diluted with ten parts of sterile salt solution. Five species of bacteria were present in approximately the same proportion as found in the stock wheat infusions. The concentration of bacteria, as checked by colony-counting methods, was about 3,000,000 per cubic centimeter (Table 1, C). This approached the equivalent of a 0.001 X concentration suspension as used by Johnson (1933, 1936) and described in the next

TABLE 5

COMPARISON OF AVERAGE TOTAL NUMBERS PRODUCED IN NONSTERILE CULTURES INOCULATED WITH VARYING NUMBERS OF
Euglena gracilis IN 1 DROP
(Average of twelve series)

Inoculated Number	Average Total Number	Comparison	Probability
1.....	6.3	1/2	0.14
2.....	8.75	1/4	0.0000
4.....	11.7	1/8	0.0000
8.....	10.75	2/4	0.08
1 and 2.....	7.75	2/8	0.048
4 and 8.....	11.2	4/8	0.18
		(1 and 2)/(4 and 8)	0.0000

section. These cultures will be designated as "nonsterile cultures," to avoid confusion with other bacterial suspensions referred to in Section VI.

In 1-drop cultures of the nonsterile series (see Table 5) euglenae divided more rapidly when seeded with two, four, or eight organisms than if but one was introduced. The results were similar to those obtained with 1-drop cultures of euglenae which had been irradiated for 2 minutes, and were opposite to those found using washed-sterile euglenae in the same volumes.

The cultures started with single individuals are most markedly altered in their division-rates in all these series: they show striking acceleration in the washed-sterile inocula and lack of maintenance of numbers in the nonsterile cultures and only a slight increase in the cultures inoculated with irradiated euglenae. Are the factors which accelerate division in small-volume cultures with smallest inocula of washed-sterile individuals absent in these nonsterile populations, or are they overborne by other factors?

Whatever the causal mechanism or mechanisms may be, the effect appears to be related to the inability of the smaller inocula, more especially of isolated euglenae, to flourish under the conditions that obtain in this so-called "nonsterile" series.

VI. COMPARISONS OF NUMBERS OF *Euglena gracilis* PRESENT AFTER 24 HOURS IN SUSPENSIONS OF CONTROLLED CONCENTRATIONS AND SPECIES OF BACTERIA

Each of the five species of bacteria which composed the natural flora of stock cultures of *E. gracilis* (either wheat or salt-solution stocks) was isolated on nutrient agar plates,

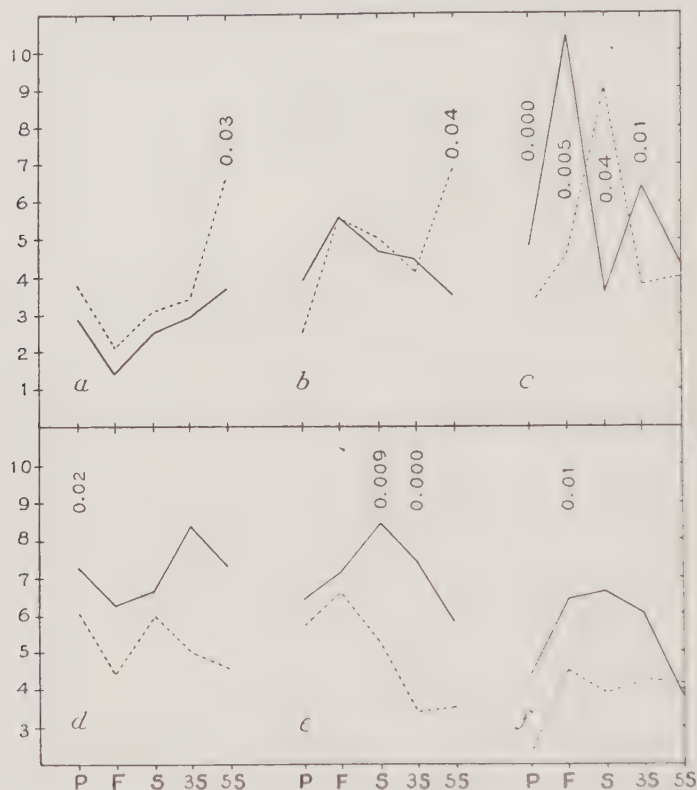


FIG. 3.—Differences in numbers of *Euglena gracilis* present after 24 hours in six concentrations of various bacterial suspensions in cultures (1 drop) inoculated with one and two (dotted lines) compared with four and eight (solid lines) washed sterile euglenae. Ordinates, average total numbers present; abscissas, types of suspension. P, *Pseudomonas*; F, *Flavobacterium*; S, *Staphylococcus*; 3S, three species suspensions; 5S, five-species suspensions. When significant, the statistical probabilities of the differences are given (see legend to Fig. 1). The various figures show different concentrations of bacteria as follows: a, X; b, 0.1 X; c, 0.01 X; d, 0.001 X; e, 0.0001 X; f, 0.00001 X.

and a platinum loopful of a 24-hour colony of each species was transferred to 10 cc. of nutrient broth and incubated 24 hours. Then a loopful was transferred from the nutrient broth suspension to 10 cc. of sterile Osterhout salt solution to compose an X concentration of a known species of bacteria. Appropriate dilution gave a 0.1 X, a 0.01 X suspension, etc.

The bacteria used were *Flavobacterium*, a pigment-producing cocci, *Staphylococcus*,

sp., and *Pseudomonas fluorescens*. The species of the last genus was definitely established. Two gram-negative, non-spore-forming rods were also in stock cultures and were used in the cultures of the five mixed species. The three species suspensions were made up of *Flavobacterium*, *Staphylococcus*, and *Pseudomonas*. For the mixed cultures, of three and five species, loopfuls from three or five of the nutrient broth suspensions were placed in 30 and 50 cc., respectively, of sterile salt solution to compose X concentrations; and appropriate dilutions were made as indicated. Euglenae, in preparation for growth in

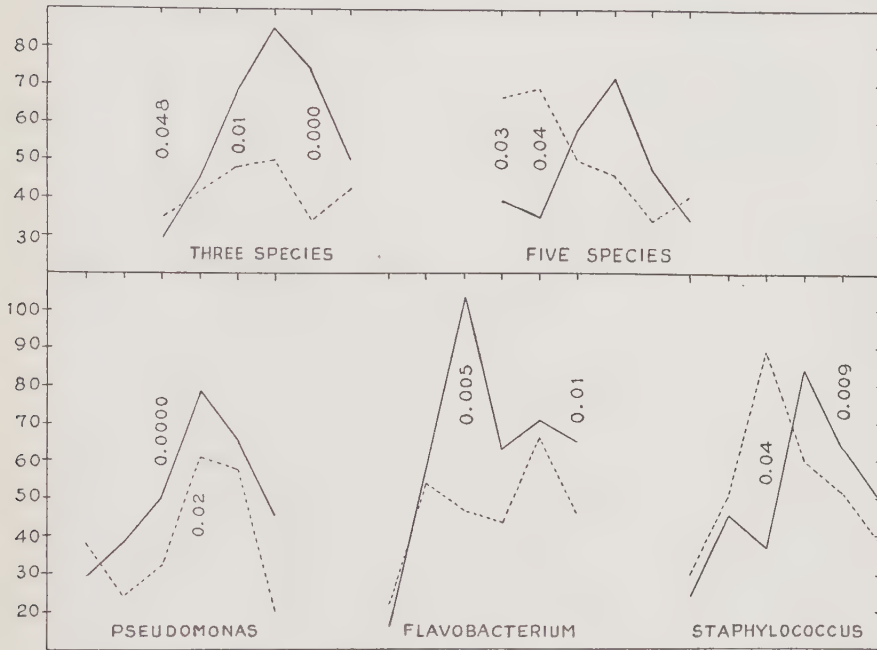


FIG. 4.—Differences in numbers of *Euglena gracilis* present after 24 hours in five bacterial suspensions (1 drop) of varying concentrations with one and two individuals in the inocula (dotted lines) compared with four and eight (solid lines). Ordinates, average numbers present; abscissas, concentrations of the suspensions for each pair of graphs; the points show the following concentrations of bacteria, X, or X, 0.01 X, 0.001 X, 0.0001 X, and 0.00001 X. When significant, the statistical probabilities of the differences are given.

these media, were passed through the three dilution baths and were mouth-pipetted to the final culture slide, as in all the washed-sterile series. All cultures were made in 1 drop of the standard salt solution.

Figures 3 and 4 schematize the cumbersome details accumulated in this series in a relatively simple fashion. The data from which these graphs are taken, together with the statistical calculations, are available, as are all the data for tables for previous sections (Sweet, 1937). Results from the cultures inoculated with one and two individuals have been considered together (dotted line), and, likewise, those inoculated with four and eight individuals (solid line).

Both figures present the same material but with different emphases. Figure 3 shows

the six concentrations separately, with the numbers of *Euglena* present after 24 hours in the five different types of bacterial suspension. Figure 4 presents the five different types of suspensions separately, with the numbers present in the various concentrations of each type of suspension. The two figures can be cross-read. The following summary of the material here presented uses both graphs.

1. There is a lack of maintenance of the numbers of *E. gracilis* inoculating a series in the majority of cases in these bacterial suspensions of different species and concentrations. In the sixty cases, only four instances occur in which an average of more than eight (the original inoculated for each series) individuals are present in these suspensions after 24 hours. Hence, for the most part we are measuring survival, not maintenance or growth of cultures.

2. From glancing at Figures 3 and 4 it is readily observed that in most cases the cultures inoculated with the greater numbers (four and eight individuals, heavy line) are capable of sustaining greater numbers than are those with fewer numbers in the inocula (one and two, dotted line).

3. This dominance of the larger-sized inocula occurs in nearly every instance in the less concentrated suspensions. (Refer to Figure 3, *d*, *e*, and *f*, the 0.001 X, 0.0001 X, and 0.00001 X concentrations.) This is true regardless of the specific type of suspension. However, this increase in numbers is significant in only four of the fourteen individual instances (Fig. 3, *d*, *Pseudomonas*; *e*, *Staphylococcus* and three species; and *f*, *Flavobacterium*).

4. In the more concentrated suspensions there is less uniformity in the results. In the 0.01 X concentration, three cases of significantly greater numbers occur in favor of the larger inoculating group, in the *Pseudomonas*, *Flavobacterium*, and three-species suspensions. The smaller inoculating group grow best in the *Staphylococcus* suspension at this concentration. Also in the 0.1 X concentration, the most significant difference occurs in favor of the smaller inoculating group in the five-species suspensions (Fig. 3, *b*).

5. In the most concentrated suspensions, X, the smaller inoculating group is consistently more numerous, but significantly so only in the five-species suspensions (Fig. 3, *a*).

6. In the most dilute and most concentrated suspensions—the X, 0.1 X, and 0.00001 X—numbers present in 24 hours are reduced more than in the moderately concentrated suspensions (Fig. 3, *a*, *b*, and *f*; cf. *c*, *d*, and *e*).

7. Hence, it is apparent that, where there are significant differences in the more concentrated suspensions, the smaller the numbers in the inocula the greater the chance of surviving; and in the less concentrated suspension, the larger the number in the inocula the greater the chance of survival. The transition occurs at the 0.01 X concentration.

8. In viewing these same relations in Figure 4, in order to note the specific effects of varying bacterial species in suspension and in a range of concentrations, no one type of suspension is much different in effect than any other. In checking the average total numbers in each suspension, *Pseudomonas* is least conducive to survival, with an average of 4.55 living after 24 hours from an initial seeding of 8.0; the five-species suspension next, 5.01; the three-species, 5.11; *Flavobacterium*, 5.33; and *Staphylococcus*, sp., the most conducive, 5.41. These differences are, obviously, not great.

9. Significant differences in survival in each of the suspensions are not common.

a) In the *Pseudomonas* suspensions, statistically significant differences in numbers

present occur in the 0.01 and the 0.0001 X concentrations in favor of larger numbers in the inocula, although in all the concentrations of this type of suspension, except in the most concentrated, the larger number in the inocula are in advance of those with smaller numbers.

b) In the *Flavobacterium* suspension, again larger numbers do best in the concentrated suspension and are significantly more successful in sustaining *Euglena* in the 0.01 and the 0.00001 X; in fact, in the 0.01 concentration, increase in numbers occurs beyond the maintenance level.

c) In the *Staphylococcus* suspensions, only one significant difference occurs, and this in favor of the cultures with one and two individuals in the inocula in the 0.01 X concentration. In this group smaller numbers in the inocula are more numerous in the three most concentrated suspensions. In the more dilute suspensions the greater numbers are in advance—significantly so in one case, the 0.0001 X concentration.

d) In the three-species suspensions, three significant differences occur in favor of larger-sized inocula, in the 0.1 X, 0.01 X, and 0.0001 X concentrations. Only in the most concentrated suspensions do the smaller-sized inocula succeed in sustaining *Euglena* better than do larger-sized inocula; but the numbers are very reduced, and the difference is not significant.

e) And lastly, in the five-species suspension the significantly greater numbers occur in the X and the 0.1 X concentrations with smaller-sized inocula; and in the less concentrated, 0.0001 X concentration, with the greater-sized inocula.

Hence, in the eleven cases of statistically significant differences in the numbers surviving in 24 hours in such bacterial suspensions, seven of them occur in cultures with greater numbers in the inocula, three instances in the 0.01 X, one in the 0.0001 X, two in 0.00001 X, and one in the 0.000001 X concentrations. The three cases in which smaller numbers in the inocula favor the survival of euglenae were in the more concentrated suspensions, X, 0.1 X, and 0.01 X. It can be said that, in general, in each of these specifically different bacterial suspensions significant differences in survival are apparently related to the numbers seeded and are somewhat more assured in cultures with greater numbers in the inocula in the moderately dilute concentrations.

The division-rates in these bacterial suspensions, when viewed as a whole, are not as great as in any other of the series studied—washed-sterile, irradiated, or even non-sterile. In fact, usually maintenance is not possible: only survival-rates can be compared. There appears to be some deterrent of division present in practically all the concentrations, species, and combined species tested. However, because rates are not lower in at least one concentration of the five types of suspension, generally the 0.01 X or 0.0001 X, than in some of the media already studied, there may be no factor except concentration of the specific bacterial suspension which is acting to lower rates.

In the five-species suspension, where the same species are present as in the stock and nonsterile cultures, it would be expected, perhaps, that in equal quantities the same or similar rates of division might obtain as in suspensions with similar proportions of the stock cultures. But numbers of bacteria present are considerably less. It appears that in equal quantities there may be some one or more species present in greater or less quantity than is conducive to growth of the culture in this short period. The *Pseudomonas* suspensions, the five- and the three-species suspensions showed least growth. Because *Pseudomonas* is common to these three least growth-promoting suspensions, there may be some effect of this species acting to deter division. More complete

study of the effect of *Pseudomonas* in mixed-population suspensions would be needed to reveal its symbiotic role; that is to say, the possible maximum or optimal numbers of *Pseudomonas* in a *Euglena* culture are not known from this limited survey.

Deterrence of division-rates was so universal as to lead one to suspect that, in the salt solution which was used, the presence of these bacteria react on *E. gracilis* adversely rather than beneficially. It is clear from these experiments that in the absence of some organic material, which is present in the wheat infusion, *Euglena* grows better in salt solution in the absence of bacteria than in the presence of the bacteria studied. The end-products of bacterial activity may not be absorbed, and the disintegration substances of dying and/or dead bacteria may be sufficient to limit division of *Euglena*.

In any event, as the numbers of introduced bacteria become smaller and conditions therefore approach those in sterile salt solutions, the effects of differential inoculation became similar to those found with the cultures in sterile salt solutions.

Also, whatever the true relationships are between *E. gracilis* and its associated bacteria in salt and wheat cultures, it is clear that in salt solutions and in the less concentrated cultures of many different types of bacterial suspensions, greater numbers in the inocula were more protective and effected greater survival. In the more dense suspensions the smaller numbers in the inocula sometimes show significantly greater numbers surviving. This latter effect may be related to the spatial factor found elsewhere in aggregation phenomena but not indicated especially in this population study heretofore. The former effect may be due to the greater capacity of increased numbers to counteract the toxicity of such bacterial suspensions in salt solution and of some of the toxic effects of plain salt solutions in large volumes of cultures made relatively free from bacteria by dilution methods as reported in Section III and by irradiation as shown in Section IV.

VII. THE EFFECTS OF NUMBERS ON SURVIVAL OF *Euglena gracilis* SUBJECTED TO ULTRA-VIOLET IRRADIATION

The methods employed for these cultures was that of transferring the desired number of euglenae after 20 minutes in dilution Bath C into open depression slides in 4 drops of sterile wheat solution, or bacteria-rich wheat solution, or into 1 or 4 drops of sterile salt solution. They were then subjected, as was described previously, for varying lengths of time to the same type of ultra-violet irradiation. As usual, results are based on counts made at the end of 24 hours. The solution for the sterile wheat was prepared by using boiled wheat after it had stood 24 hours, as described in Section II. The bacteria-rich wheat suspensions were similar to the type used in Section V with 1 cc. on the supernatant fluid of centrifuged *Euglena* wheat infusions mixed with 10 cc. of sterile wheat solution.

The protection of numbers of *E. gracilis* is apparent in the differences with which individuals are able to withstand the deleterious effects of long exposure to ultra-violet rays (Fig. 5). The presence of organic compounds and of bacteria definitely contributes to the survival-time of *E. gracilis* in sterile and bacteria-rich wheat infusions, particularly when ten euglenae are present and when twenty and forty are present through 6 minutes' exposure; beyond that time this differential was not present.

Cultures with single individuals in 4 drops of any of the three solutions were all killed after no more than $4\frac{1}{2}$ minutes' exposure. Those with ten euglenae survived the

6½ minutes' exposure in sterile wheat and bacteria-rich wheat solutions, whereas those in sterile salt solution survived no longer than did isolated individuals. Cultures with twenty individuals were able to live in the sterile salt solution after a 7-minute exposure; and in the wheat, sterile, and bacteria-rich, after an 8-minute exposure. Forty euglenae in 4 drops withstood exposures of 7½ minutes in sterile and bacteria-rich wheat solutions; in sterile salt solution the majority died at the end of 6 minutes' exposure, although a few persisted after 8 minutes. This is the only medium which allowed such survival after long exposure.

When euglenae are exposed in sterile salt-solution cultures of only 1 drop, the dele-

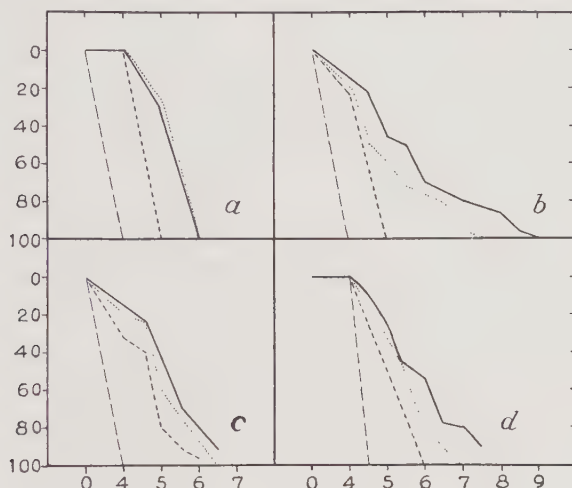


FIG. 5.—Average numbers of *Euglena gracilis* present after 24 hours, in three types of culture media, after exposure to ultra-violet irradiation of varying durations. (a) One drop, sterile salt solution. (b) Four drops, sterile salt solution. (c) Four drops, sterile wheat solution. (d) Four drops, bacteria-rich wheat suspension. Ordinates, percentage of loss; abscissas, duration of irradiation in minutes. . . . = single euglenae; --- = ten euglenae; -- = twenty euglenae; — = forty euglenae.

terious effect of irradiation is greatest; and only cultures with twenty and forty organisms survive a 6-minute exposure; those inoculated with one or ten, only 4 minutes.

This small group of comparisons shows the advantage afforded greater numbers in any of these media in withstanding the effects of long exposure to ultra-violet irradiation and the relatively beneficial effects of organic media during all except the longer exposures. In the cases of twenty and forty euglenae in the bacteria-rich media, the presence of bacteria as an added protection against the toxicity of the rays is apparent. In the smaller volume of sterile salt solutions the deleterious effects are more drastic; even in these an increase in numbers of euglenae present insures more protection.

These relations cannot be explained on the basis of the amount of penetration, in so far as volume and bacterial effects are concerned. The presence of one or of forty euglenae in 1 or 4 drops of media, with or without bacteria or their end-products, would make very small difference in the penetration of the ultra-violet rays; hence, this effect is probably caused by some other set of relationships. Since the euglenae were cultured in the same solutions in which they were exposed to ultra-violet irradiation and since

irradiated water normally has an increase in ozone, it may well be that we are dealing here simply with mass protection from ozone. Euglenae are not particularly favorable organisms for a further analysis of the mechanism of this protection. Eliminating ozone as a factor, using the planarian, *Euplanaria dorotocephala*, Allee and Wilder (1939) have demonstrated mass protection from ultra-violet irradiation and have made some progress with an analysis of protective factors.

VIII. GENERAL DISCUSSION AND SUMMARY

Cultures in inorganic salt solution, almost or quite bacteria-free, in various suspensions of bacteria, in sterile and bacteria-rich wheat infusions, and of volumes ranging from 1 to 40 drops, have been used in this study of populations of *E. gracilis*. With numbers present at the end of 24 hours as an index, a variety of differences in division-rates of cultures inoculated with one, two, four, and eight individuals have been analyzed in order to find the effect of numbers used in initial seedings on survival and population growth. Constancy of light, temperature, humidity, pH, and in most cultures a strictly autotrophic media have been maintained. The controlled variables include, in addition to the number of euglenae inoculated, kinds and numbers of bacteria, volume, including surface area, and two methods of securing bacteria-free cultures. All the individuals used have been members of two clones between which and within which no measured differences in physiological states or division rates have been significant.

With such relatively small subcultures the results obtained indicate:

1. Greater rate of growth with smaller inocula:
 - a) Washed-sterile euglenae in 1 (0.05 cc.) to 4 (0.20 cc.) drops
 - b) Irradiated-sterile euglenae in 4 drops
2. Greater maintenance with smaller inocula:
 - a) In bacterial suspensions
 - (1) 0.01 X concentration, *Staphylococcus* suspensions
3. Greater survival with smaller inocula:
 - a) In more concentrated bacterial suspensions
 - (1) X concentration, five-species suspensions
 - (2) 0.1 X concentration five-species suspensions
 - (3) 0.1 X concentration
1. Greater rate of growth with larger inocula:
 - a) Washed-sterile euglenae in 5 (0.25 cc.) drops
 - b) Irradiated-sterile euglenae in 1 drop
 - c) Nonsterile cultures of 1 drop
2. Greater maintenance with larger inocula:
 - a) In bacterial suspensions
 - (1) 0.01 X concentration, *Flavobacterium* suspensions
3. Greater survival with larger inocula:
 - a) Washed sterile euglenae in 10, 20, and 40 drops
 - b) In more concentrated bacterial suspensions
 - (1) 0.01 X concentration three-species suspensions
 - (2) 0.01 X concentration, *Flavobacterium* suspensions
 - (3) 0.01 X concentration, *Pseudomonas* suspensions
 - c) In less concentrated bacterial suspensions
 - (1) 0.001 X concentration, *Pseudomonas* suspension
 - (2) 0.0001 X concentration *Staphylococcus* suspensions

- (3) 0.00001 X concentration *Flavobacterium* suspensions
- (4) 0.0001 X concentration, three-species suspensions
- d) With exposure to toxic durations of ultra-violet irradiation
 - (1) In 1 and 4 drops of sterile salt solution
 - (2) In sterile wheat solution
 - (3) In bacteria-rich wheat suspensions

From these results in bacteria-free cultures it is apparent that density of inocula markedly affects growth and/or survival of populations of *E. gracilis*. Also, it seems clear that there exist optimal, suboptimal, and supraoptimal volume relations in cultures with washed-sterile euglenae which regulate growth or survival, and, also, that there are optimal, suboptimal, and supraoptimal densities of the bacteria associated with *Euglena*. For the most part, the mere presence of bacteria in a salt solution deters the development of euglenae. However, some materials or material are present in wheat media, even when autoclaved, which is accelerative to division-rates and which also affords protection against the toxic effects of ultra-violet irradiation.

Frequently during the experiments reported here, the numbers of individuals in the inoculum of a culture have made a significant difference in the rates of division and abilities to withstand environmental exigencies. As the foregoing summary shows, the smaller inocula have the advantage in a number of instances: with washed-sterile euglenae in 1 to 4 drops regardless of identical volume relations; in the cultures with larger volumes started with irradiated euglenae; and in the more concentrated bacterial suspensions. Stimulation due to the absence of members of the same species, and/or absence of a larger quantity of overly toxic substance or substances or of deleterious physical conditions, may determine the reactions of smaller numbers in the first instances. With the denser bacterial suspensions it is possible that survival is greater because of less competition in these more-dense mixed populations for space, light, acids, gases, or for certain of these.

Instances in which a larger inoculum has significant advantages over smaller inocula appear to be more numerous under the conditions tested. When the defects of an inorganic media accumulate, as in the volumes of from 5 to 40 drops; when toxic effects of irradiation are increased in smaller volumes, owing to greater evaporation, etc., as in the 1-drop versus 4-drop cultures; when time of exposure to ultra-violet irradiation is increased to a toxic amount; and when moderate and more dilute concentrations of various species of bacteria are present, greater numbers in the inocula insure cultures of having greater reproductive rates or greater chances of survival, or both.

There is no evidence from these experiments to indicate whether the irradiated-sterile, the washed-sterile, or nonsterile euglenae are most nearly normal. We know that in nature *Euglena* is an autotroph of stagnant, fresh-water, unshaded pools. In view of the type of reaction which occurs in the presence of mixed bacteria (of these relatively dense concentrations) and in the presence of a toxic agent (as represented by irradiation), protection as a result of the presence of greater numbers in the inoculum undoubtedly could occur in nature. Likewise, in the absence of bacteria, and with too great toxicity of the media, the accelerated division-rate of reduced numbers in the in-

oculum of *E. gracilis* may occur. That the types of population physiology shown in these cultures may exist in nature in a similar manner and not be limited to a few laboratory setups is not, therefore, unreasonable.

The experiments were not planned, however, to duplicate nature. They were conducted in the manner here recorded in an effort to bring the complex conditions to be found in an ordinary laboratory infusion under at least partial experimental control. These analytical experiments were attempted only after a long series of tests with more complex, and therefore less closely controlled, conditions which yielded indefinite, erratic results.

There have been described in this paper a number of situations, but there has been no attempt to analyze the causal factors at the base of such relationships as exist in these relatively simple experimental populations. The results of this study are relatively consistent within themselves and with the findings of others. Some of the complexities involved in this paper are, a priori, confusing. Errors in reasoning and in the conclusion based on this work may possibly be due to the natural tendency to simplify the categories and so avoid apparent inconsistency, in place of allowing many observations to dangle unrelated.

Similar work with protozoans has been done mainly with ciliates; and the relations obtaining in members of that group, fully discussed and reviewed by Allee (1931, 1934, 1935) and Johnson (1937), do not necessarily hold with an autotrophic organism. A discussion of the similarities and differences in results dependent on different physiological systems is reserved for the present.*

The recent studies of Mast and Pace (1938) with the flagellate, *C. paramecium*, and of Reich (1938) with a soil amoeba, *M. palestinensis*, present additional substantiation of an allelocatalytic effect, whatever its basic factors may be. According to Reich (1938, p. 357), "cultures whose initial population was less than 0.03 amoebae per 0.1 cu. mm. of solution showed a distinctly lower rate of division in the first days than the controls with higher initial populations." And Mast and Pace (1938, p. 380) find "the rate of reproduction of *Chilomonas* increases to a maximum as the volume of culture fluid per individual decreases and then decreases to zero." The results obtained in various modifications of their experiments strongly indicate that *Chilomonas* secretes a substance which favors growth which they call X.

The only population study with a green flagellate that was available when the present paper was written was that of Jahn (1920), who found that the density of *E. gracilis* at the beginning of mass cultures which favor greatest growth was to be found in initial concentrations ranging from 38 to 39 organisms per cubic centimeter, rather than in those with a greater density of 350 to 804 organisms per cubic centimeter. In his work it is clear that "fewer" numbers in the inocula are most favorable in the early development of large quantity *Euglena* cultures.

It may be said that the conditions of his experiments were so different from those herein described as to make a comparison of results impossible.⁶ And so it may be, but it is interesting to note certain similarities in results which do appear. If we analyze the terms "small" and "large" seedlings in both series, we find a basis for comparison which may shed some light on Jahn's results and those of the present work.

*The composition of the media used by Jahn contained hydrolyzed casein and different inorganic salts; surface-area relations of mass cultures are different from those of depression-slide cultures.

In the present study of small-volume cultures the greatest concentration of organisms inoculating a culture was that of eight individuals in 1 drop, which is approximately 160 individuals per cubic centimeter. The concentrations which yielded greatest rates were those of one and two individuals per 4 drops (or five and ten organisms per cubic centimeter) and those of four and eight individuals per 5 drops (or sixteen and thirty-two organisms per cubic centimeter). The most dilute concentration of initial population was one organism per 40 drops or one-half organism per cubic centimeter. In other words, the greatest division-rates obtained in this range of small-volume cultures occurred in the moderately dense seedings. A comparison of Jahn's findings with a part of the data at hand (those with washed-sterile euglenae in salt solutions) is possible in this form.

A. Jahn's mass cultures	Cases in which less concentrated inocula gave a greater division-rate than more concentrated: 1. 89 individuals per cubic centimeter, compared with 890 individuals per cubic centimeter, showed a 10:1 division-rate 2. 55 individuals per cubic centimeter, compared with 555 individuals per cubic centimeter, showed a 3:1 division-rate
B. Present depression-slide cultures	Cases in which less concentrated inocula gave a greater division-rate than did more concentrated inocula: 1. 20 individuals per cubic centimeter (one individual per 1 drop), compared with 160 individuals per cubic centimeter (eight individuals per 1 drop), showed a 12.6:9.8 division-rate 2. 5 individuals per cubic centimeter (one individual per 4 drops), compared with 40 individuals per cubic centimeter (eight individuals per 4 drops), showed a 27.0:16.0 division-rate Cases in which more concentrated inocula gave a greater division-rate than did less concentrated inocula: 1. 160 individuals per cubic centimeter (eight individuals per 1 drop), compared with 8 individuals per cubic centimeter (eight individuals per 20 drops), showed a 9.8:3.25 division-rate 2. 40 individuals per cubic centimeter (eight individuals per 4 drops), compared with 4 individuals per cubic centimeter (four individuals per 20 drops), showed a 10.3:1.25 division-rate

By combining the present findings with those of Jahn, it is possible to suggest that a supraoptimal density of initial populations of washed-sterile *E. gracilis* ranges above 80 individuals per cubic centimeter. An optimal size of inoculum apparently lies between 4 and 50 individuals per cubic centimeter, and a suboptimal one contains still fewer euglenae. This is a broad optimal range, to be sure; and it would not hold in each specific detail, particularly in depression-slide cultures, because there is a definite function of particular volumes, with an optimum at 4 and 5 drops in these experiments, regardless of initial population density. Jahn did not vary the surface area or volume of his mass cultures, so that relations which may depend on these are not brought to light.

Such a comparison as this indicates, at least, the necessity of using the terms "larger" and "smaller" with caution when referring to size of inocula, and of using, rather, the number of individuals per cubic centimeter wherever possible in drawing generaliza-

tions. In short, the densities of inocula which Jahn terms "light" are in the present work "heavy"; and the greatest division-rates occurred with approximately the same densities of initial population in both Jahn's mass cultures and the present series, in which small volumes are used.

Other functions or reactions of different numbers of the same species in the seeding of a culture appear in several ways in the data from small subcultures.

The data, as a whole, add a greater number of instances to the rapidly growing amount of verification of the principle of automatic co-operation among all plant and animal life than to the older and generally more easily proved interorganismic effects associated with overcrowding. Thus, these experiments lend additional support to the idea of the existence of an automatic mutualism, which Allee (1938) and his associates are investigating.

That signs of the beginnings of automatic co-operation and, to that extent, of a meager social organization appear in these unicellular and solitary chlorophyll-bearing organisms, whose status as members of the plant or animal kingdom is still in dispute, is not strange in view of the fact that colonial organization and even sexual differentiation are also to be found in the closely related *Phytomonadida*.

IX. CONCLUSIONS

1. Optimal, supraoptimal, and suboptimal volume relations of cultures of washed-sterile euglenae exist, which regulate growth or survival of a group accordingly.

2. There are optimal, suboptimal, and supraoptimal densities of bacteria associated with euglenae populations. Deterrence of division is common in the densities and with the species of bacteria tested.

3. Some material or materials present in sterile or nonsterile wheat infusions is more conducive to growth of populations of euglenae, and also affords more protection against toxic effects of ultra-violet irradiation, than does the artificial salt solution tested.

4. Smaller inocula show significantly greater advantage in situations in which the deleterious elements of the environment are not too great.

5. Larger inocula show significantly greater advantage when the defects of the environment are greatest.

6. Jahn (1929) found, from the study of mass cultures, no evidence of the Robertson effect; his least-dense cultures, however, correspond fairly well to the optimal numbers constituting an inoculum for the present small volume cultures, whose division-rates were found to be greater than either those in more dense or more dilute cultures.

7. The Robertson effect, or certain aspects of it, is present in some of the optimal volumes and may be operating in all of the supraoptimal volumes reported in this paper.

8. To this extent the present studies yield evidence of Robertson's effect. There is, however, no clear evidence of the validity of his theory.

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